Evaluation of Transforming Growth Factor β and Type I Procollagen Gene Expression in Fibrotic Skin Diseases by In Situ Hybridization

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Full thickness biopsies of affected skin and fascia from one patient with diffuse fasciitis and eosinophilia (DF), two patients with generalized morphea (GM), and five patients with progressive systemic sclerosis (PSS) of recent onset were examined for the expression of transforming growth factor β_1 (TGFβ_1) and type I procollagen genes by in situ hybridization with human sequence-specific cDNA. An increased number of fibroblasts showing clearly detectable expression of proα1(I)collagen gene was found in all fibrotic lesions when compared with unaffected skin from the patient with DF and skin from two normal individuals examined in parallel. Expression of the TGFβ_1 gene was noted in a fibroblast subpopulation of the affected tissues from the patients with DF and GM. In contrast, the TGFβ_1 gene was not expressed at a detectable level in affected skin from the five patients with PSS. The results suggest that TGFβ_1 may play a role in the development of skin fibrosis in cases of DF and GM. However, from these studies, we cannot implicate TGFβ_1 in the pathogenesis of skin fibrosis in PSS. *J Invest Dermatol 94:365–371, 1990*

Several cutaneous diseases are characterized by excessive and frequently progressive fibrosis of the dermis and subcutaneous tissues. Prominent among these disorders are progressive systemic sclerosis (PSS) and generalized morphea (GM), as well as the recently described syndrome of diffuse fasciitis with eosinophilia (DF), also known as Shulman's syndrome [1–3]. The hallmark of the pathologic alterations in these disorders is the excessive deposition of collagen and other connective tissue macromolecules in the dermis and/or the subcutaneous and fascial structures, often accompanied by variable degrees of chronic inflammatory cell infiltrates. The proximity of fibroblasts and chronic inflammatory cells in tissues undergoing active fibrogenesis has suggested that cytokines or other diffusible products of the inflammatory cells may play an important role in the development of the excessive fibrosis observed in these diseases [4].

Transforming growth factor β_1 (TGFβ_1), a cytokine produced by many neoplastic cells and by most normal cells, including macrophages and lymphocytes [5], has been implicated as a possible mediator that may play a role in the pathogenesis of various fibrotic diseases [6]. TGFβ_1 is a potent chemotactic agent for monocytes and fibroblasts [7,8]. In addition, TGFβ_1 causes marked stimulation of collagen and fibronectin production by fibroblasts in vitro [9,10] and accelerates wound healing in experimental animals [11,12]. Studies on cultured fibroblasts have shown that the effects of TGFβ_1 are accompanied by elevated steady-state levels of collagen and fibronectin mRNA [13–15], which persist even after removal of TGFβ_1 from the culture medium [14]. It appears, therefore, that TGFβ_1 can cause a prolonged stimulation of fibroblast connective tissue biosynthesis and may, thus, play a significant role in the development of fibrosis.

The purpose of this study was to identify and localize the cells expressing TGFβ_1 and type I procollagen genes in samples of affected tissues from patients with PSS, GM, and DF employing in situ hybridizations with human sequence-specific cDNA. We found that fibroblasts in the affected skin from patients with these three diseases displayed elevated expression of proα1(I)collagen gene compared to normal skin. In situ hybridizations with a human TGFβ_1 cDNA showed clearly detectable levels of TGFβ_1 mRNA in the affected dermis and fascia of patients with GM and DF. However, the skin samples from the five PSS patients examined simultaneously failed to show detectable dermal expression of the TGFβ_1 gene. These results suggest that there may be differences in the

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Abbreviations:

cDNA: complementary deoxyribonucleic acid
DF: diffuse fasciitis and eosinophilia; Shulman's syndrome
DNA: deoxyribonucleic acid
EDTA: ethylenediaminetetraacetic acid
GM: generalized morphea
mRNA: messenger ribonucleic acid
PSS: progressive systemic sclerosis; systemic sclerosis
Scl-70: scleroderma 70 antibodies
TGFβ_1: transforming growth factor β_1

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pathogenetic mechanisms responsible for the overproduction of collagen in PSS and the two other cutaneous fibrotic disorders studied here.

MATERIALS AND METHODS

Patients Five patients with PSS of recent onset (less than 12 months) and rapid progression of skin sclerosis as described recently [16], were studied. All patients fulfilled the American Rheumatism Association criteria for diagnosis of PSS at the time of skin biopsy [17]. Two patients with GM with lesions of recent onset and one patient with the classic features of Shulman's syndrome, including peripheral blood eosinophilia, were also studied. Normal skin tissue was obtained from the unaffected forearm of the patient with DF and from two normal individuals.

The clinical features of the cases studied are described in Table I. None of the patients were receiving corticosteroids, immunosuppressive agents, or other therapy known to affect collagen metabolism at the time the biopsies were obtained. Antinuclear antibodies were demonstrated by immunofluorescence in the sera of the five PSS and the two GM cases in titers ranging from 1 : 160 to 1 : 2,560. Antitopoisomerase I (Scl-70) antibodies were present only in two PSS cases (cases 4 and 5). Anticentromere antibodies were absent in all cases. No antinuclear antibodies were detected in the patient with DF.

In Situ Hybridization Full-thickness excisional skin biopsies from the dorsal forearm of the PSS patients and the two normal individuals, from the involved skin of the GM and DF patients, and from the uninvolved contralateral area of the DF patient were obtained as described previously [18]. Five-μm-thick cryosections were cut from snap-frozen biopsy samples and fixed immediately with fresh 4% paraformaldehyde in phosphate-buffered saline for 20 min. The samples were then pre-treated as described previously [19]. Immediately before hybridizations, the samples were heated at 90°C for 5 min, and cooled rapidly on ice and were hybridized for 16 h at 42°C in a solution containing 0.1 μg/ml ^{32}P-labeled cDNA probe, 50% formamide, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.6 M NaCl, 10% (wt/vol) dextran sulphate, 200 μg/ml denatured and sheared salmon sperm DNA, 0.5 mM EDTA, 0.02% (wt/vol) ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, and 10 mM Tris-HCl, pH 7.4. After the hybridization, the samples were washed as described [19], the final stringency being 0.2 × SSC at 42°C.

The [^{32}P]cDNA-mRNA hybrids were detected by immersing the samples into Kodak NTB-3 autoradiography emulsion (Eastman Kodak, Rochester, NY) diluted with an equal volume of 0.6 M ammonium acetate, and exposing them in a desiccant-containing box for 5 d at 4°C. The samples were developed with Kodak D-19 developer, stained with hematoxylin, and dehydrated with ethanol.

cDNA Probes The following human sequence-specific cDNA were utilized in this study: for type I collagen, a 1.8-kb proα1(I)collagen cDNA (Hf 677) [20]; for type II collagen, a 525-bp proα1(II)collagen cDNA (pHCAR1) [21]; for TGFβ_1, a 1.9-kb cDNA [22], kindly supplied by Dr. R. Derynck (Genentech, South San Francisco, CA).

RESULTS

Histologic Features All skin biopsies were examined after staining with hematoxylin-eosin and Masson's trichrome blue. The samples from all PSS patients uniformly displayed increased dermal thickness with accumulation of thick bundles of collagen extending into the adipose tissue and encasing eccrine sweat glands and hair follicles. Fibroblasts were found scattered throughout the dermis but were more abundant in the papillary dermis and near the dermis-adipose tissue interface. Isolated foci of inflammatory cells were observed, particularly in areas surrounding skin appendages and in the deeper zones of the dermis. Most of the inflammatory cells were small lymphocytes, but macrophage/monocytes and plasma cells were also present. Trichrome blue staining of the tissues demonstrated extensive accumulation of collagen extending from the papillary dermis to the superficial layers of the adipose tissue.

In the biopsy of affected skin and fascia from the patient with DF, the epidermis and the papillary dermis appeared normal. Abundant thick collagen fibers were seen near the adipose tissue and extending into the fascia. The fascia was markedly thickened and displayed accumulation of collagen and the presence of an intense inflammatory cell infiltrate consisting of mononuclear cells and few eosinophils. The inflammatory cells were distributed diffusely throughout the fascia and the lower layers of the dermis. Moderate perivascular infiltrates without vessel wall necrosis were also present.

The skin biopsies of the two GM cases had a histologic appearance similar to that of DF, including extensive accumulation of connective tissue in the fascia, the adipose tissue, and the deeper layers of the dermis. In one of the cases, the fibrotic replacement was very pronounced and involved the entire thickness of the dermis. In both cases marked inflammatory cell infiltrates in the fascia and the adipose tissue, extending along fibrous tracts into the papillary dermis were present. However, in contrast to the findings in DF tissues, no eosinophils were present. The non-affected skin of the patient with DF showed entirely normal histologic architecture.

Expression of the Proα1(I)Collagen Gene When tissues from the five patients with PSS, the two patients with GM, and the affected skin from the patient with DF were examined by in situ hybridization with a human proα1(I)collagen cDNA, numerous fibroblasts containing radiolabeled cDNA-mRNA hybrids were demonstrated in all cases (Figs 1–3). The distribution of fibroblasts displaying positive hybridization with the proα1(I)collagen cDNA was not uniform, however, since many unlabeled fibroblasts were present throughout the tissue and occasionally in close proximity to fibroblasts with strongly positive hybridization signals (Figs 1–3).

The topographic distribution of fibroblasts displaying a positive hybridization with the proα1(I)collagen cDNA was different between the skin samples from PSS patients and those from patients with GM or DF. In the biopsies from patients with PSS, elevated expression of type I collagen was observed in fibroblasts scattered diffusely throughout the dermis, although they were more abundant in the sub-epidermal layers and in the deep dermis immediately adjacent to the adipose tissue (Fig 1*A–C*). In contrast, in the patient with DF, most of the labeled fibroblasts were localized in the fascia and in the adipose tissue, although some were present along the tracts of fibrous tissue extending into the lower part of the dermis (Figs 2*C* and 3*A*). Only a few cells with positive hybridization signal could be demonstrated in the sub-epidermal regions (Fig 2*A*). The samples obtained from the patients with GM displayed a pattern similar to that observed in DF with most of the fibroblasts showing clearly detectable expression of the proα1(I)collagen gene being localized in the adipose tissue, the fascia and the lower dermis (not shown). We did not detect a preferential accumulation of fibroblasts with positive hybridization signals in perivascular regions or inflammatory foci in any of the samples examined. To confirm the specificity of the hybridization, parallel in situ hybridizations were carried out with the cartilage-specific proα1(II)collagen cDNA. As expected from the absence of type II procollagen transcripts in non-cartilaginous tissues [23], all samples failed to show autoradiographic grains above those in the background. The samples of normal skin and the sample from non-affected skin from the patient with DF examined in parallel contained only few fibroblasts expressing detectable levels of the proα1(I)collagen gene.

Expression of the TGFβ Gene In order to examine if the expression of the proα1(I)collagen gene was accompanied by detectable levels of TGFβ_1 transcripts, we performed in situ hybridization utilizing a cDNA for human TGFβ_1. Despite extensive and careful search, none of the samples from the patients with PSS displayed detectable hybridization in the dermis or adipose tissue (Fig 1*D*). The same specimens showed autoradiographic grains in epidermal cells (Fig 1*D*). The significance of this finding, however, remains uncertain, because it may represent either expression of TGFβ_1, transcripts by epidermal cells, or non-specific binding of the probe

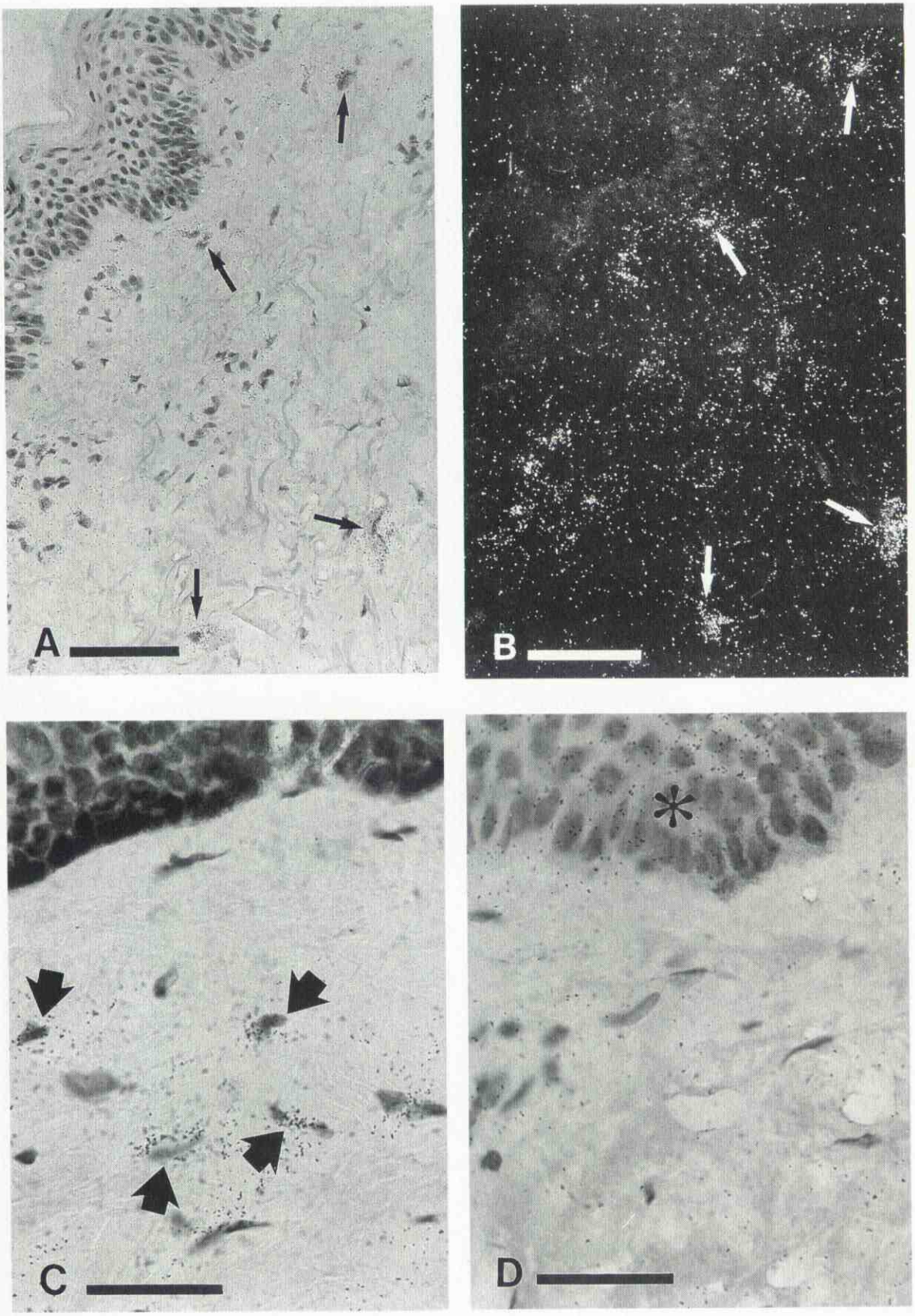


Figure 1. In situ hybridization of PSS skin samples with type I procollagen cDNA (*A–C*), or with TGFβ_1 cDNA *(D)*. The specific activity of the proα1(I) collagen cDNA was 7.5×10^8 cpm/μg and the specific activity of the TGFβ_1 cDNA was 1.15×10^9 cpm/μg. The length of exposure was 5 d. Note the presence of autoradiographic grains representative of [^{32}P]cDNA/mRNA hybrids on scattered fibroblasts (*arrows: A*, bright field; *B*, dark field). Higher magnification *(C)* reveals the cytoplasmic location of these grains. Most of the grains are detected in the immediate proximity of cell nuclei which are visualized with hematoxylin counterstain. ^{32}P-labeled TGFβ_1 cDNA/mRNA hybrids are detected in epidermis (asterisk), but not in dermal fibroblasts *(D)*. *Bar:* 100 μm in *A* and *B;* 50 μm in *C* and *D*.

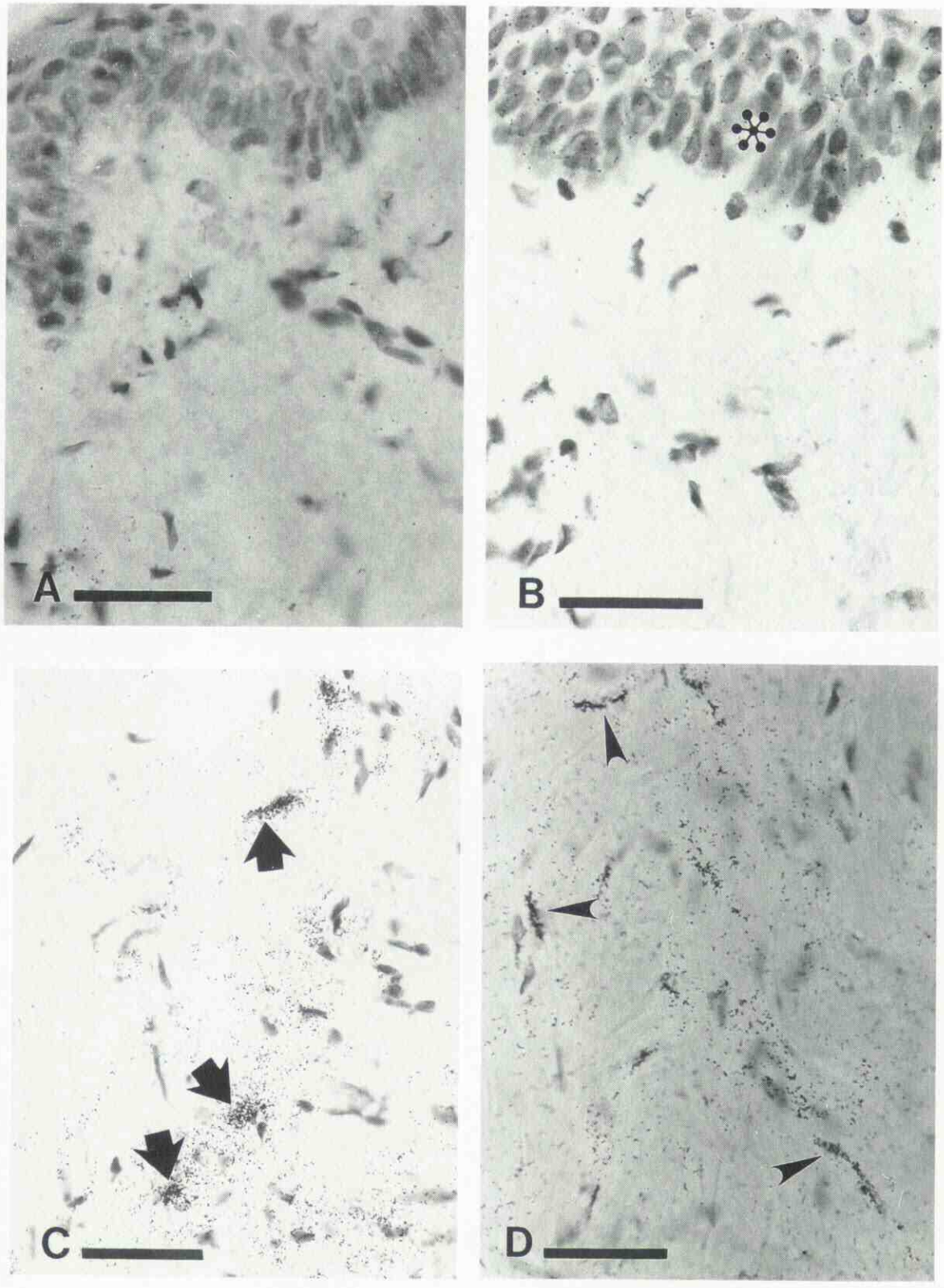
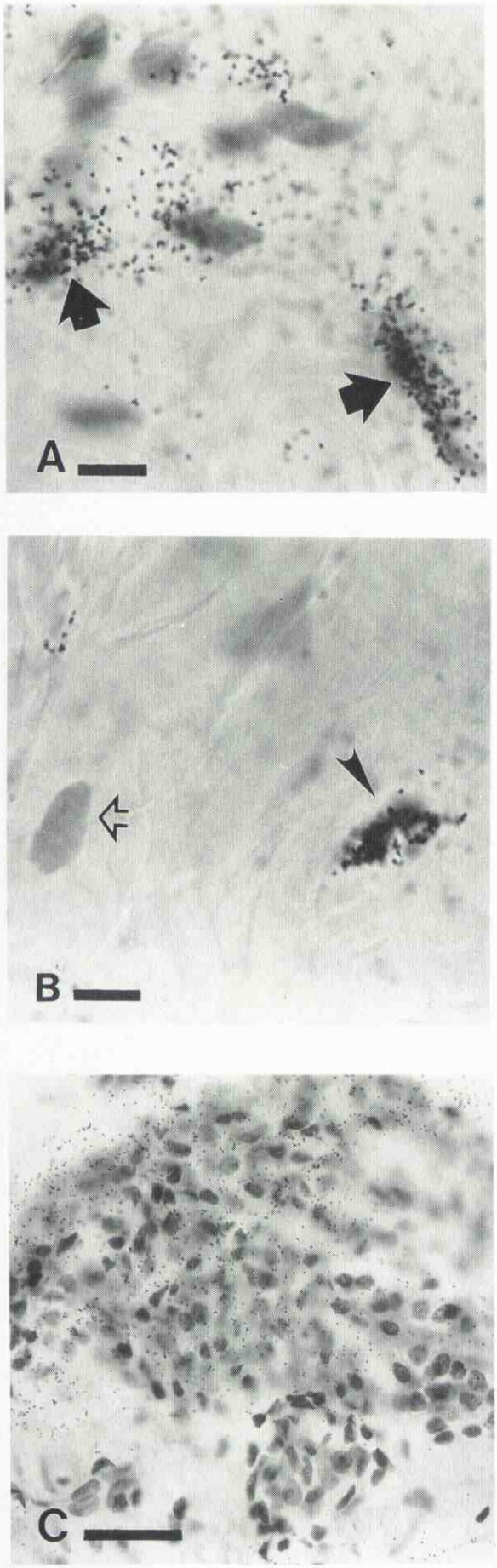


Figure 2. In situ hybridization of DF skin samples with a cDNA for proα1(I) collagen (*A* and *C*), or with a TGFβ_1 cDNA (*B* and *D*). The specific activities of the cDNA and the length of exposure were the same as in Fig 1. The presence of proα1(I) collagen mRNA is demonstrated preferentially in cells scattered in the deep dermis *(C, arrows)*. TGFβ_1 mRNA is demonstrated in cells on the same lesional locations as type I procollagen mRNA *(D, arrowheads)*. Papillary dermis is relatively free from type I procollagen mRNA *(A)* or TGFβ_1 mRNA transcripts *(B)*. Note moderate hybridization signals with TGFβ_1 cDNA in the epidermis (asterisk) *(B)*. *Bar:* 50 μm.



to epidermal cell layer components. In striking contrast to the PSS samples, affected skin from the patient with DF demonstrated numerous cells displaying intense hybridization signals with the TGFβ_1 cDNA (Figs 2*D* and 3*B*). A careful analysis of their appearance revealed that, in addition to mononuclear cells (Fig 3*C*), many fibroblasts were clearly positive for the TGFβ_1 signals (Figs 2*D* and 3*B*) and were particularly abundant in the deep dermis and at the interface between the fascia and the adipose tissue. There was no preferential accumulation of TGFβ_1-positive cells in perivascular areas, although a larger number of cells with positive hybridization were found in areas of intense inflammation. The hybridization observed in the tissues from the two patients with GM was similar to that of the patient with DF demonstrating numerous mononuclear cells and fibroblasts yielding positive TGFβ_1 hybridization (not shown) with similar topographic distribution to that of cells showing positive signals for proα1(I)collagen mRNA. The non-affected skin from the patient with DF and the two normal skin samples examined in parallel displayed the presence of autoradiographic grains only in epidermal cells (not shown).

DISCUSSION

The results described here demonstrate increased type I procollagen mRNA levels in affected skin of patients with PSS, DF, and GM compared to normal skin when examined by in situ hybridization with a human sequence-specific proα1(I)collagen cDNA. These results are in agreement with previous findings of increased expression of collagen genes as demonstrated by in situ hybridization of affected tissues from patients with PSS [24] and GM [25]. Furthermore, in agreement with these studies [24,25], we found that the distribution of fibroblasts yielding positive hybridization signals in the dermis was not homogeneous. In fact, only a sub-population was clearly positive for expression of type I procollagen mRNA while numerous fibroblasts, often in close proximity to the ones displaying elevated levels of type I procollagen mRNA, were negative.

A striking finding in our study was the intense in situ hybridization demonstrated in DF and GM tissues utilizing the human TGFβ_1 cDNA (Figs 2*D* and 3*B,C*). In addition to lymphocytes, numerous cells with the fusiform and elongated shape characteristic of fibroblasts displayed large numbers of autoradiographic grains overlaying their cytoplasms (Fig 3), indicating the in situ production of TGFβ_1 by fibroblasts. This observation lends support to previous studies [26] that demonstrated TGFβ_1 production in cultured fibroblasts following their exposure to TGFβ_1 in vitro.

In contrast to the findings with DF and GM tissues, we failed to detect positive TGFβ_1 hybridization in the dermis or adipose tissue in any of the samples from patients with PSS despite extensive and careful search. Three alternative possibilities can be proposed to explain these findings. The first is that TGFβ_1 is not involved in the overproduction of type I procollagen in PSS, but, instead, intrinsic abnormalities in the regulation of fibroblast collagen gene expression or their stimulation by other TGFβ species or by factors distinct from TGFβ_1 are responsible. The second possibility is that TGFβ_1 may have initiated the fibrotic process and caused a permanent or persistent alteration in fibroblast biosynthetic activity, but at the time the biopsies were obtained, its expression was no longer detectable in the affected dermis. We should point out, however, that the skin biopsies from the patients with PSS were obtained within 12 months of the onset of clinically apparent skin involvement. Furthermore, the clinical evaluation of these patients indicated that all

Figure 3. In situ hybridization of deep lesional dermis from the patient with DF. The specific activities of the cDNA and the length of exposure were the same as in Fig 1. High magnification reveals the presence of proα1(I) collagen *(A; arrows)* and TGFβ_1 *(B; arrowhead)* mRNA in association with a subpopulation of fibroblasts. Note one adjacent fibroblast *(B; open arrow)* without hybridization signals for TGFβ_1 mRNA. TGFβ_1 mRNA was also detected in mononuclear infiltrates in the lesional area *(C)*. *Bar:* 10 μm in *A* and *B;* 50 μm in *C*.

Table I. Clinical Characteristics of Patients Studied

Patients	Age (years)	Sex	Skin involvement: Duration (months)	Skin involvement: Progression[a]	System involvement: Raynaud's phenomenon	Musculoskeletal[b]	Gastrointestinal[c]	Pulmonary[d]	Cardiac[e]	Renal[f]
Progressive systemic sclerosis										
1.	49	M	9	19	+	+	+	+	−	+
2.	40	F	4	20	+	−	+	−	−	−
3.	39	M	5	42	+	+	+	−	−	−
4.	35	F	5	43	+	+	−	−	−	−
5.	41	F	11	50	+	−	+	−	+	+
Fasciitis with eosinophilia										
1.	34	F	5	4	−	−	−	−	−	−
Generalized morphea										
1.	35	F	2	4	−	−	−	−	−	−
2.	30	F	3	10	−	−	−	−	−	−

[a] Increase in extent of skin sclerosis (% of body surface affected) during 6 months preceding skin biopsy.
[b] Arthritis and/or flexion contractures, tendon friction rubs, myopathy, and distal phalangeal resorption.
[c] Esophageal dysmotility, and/or radiologic evidence of gastric and small and large intestine alterations.
[d] >30% decrease in DLCO, and/or restrictive changes and/or radiologic evidence of bibasilar pulmonary fibrosis.
[e] Pericarditis, and/or congestive heart failure, arrhythmias and conduction defects.
[f] Malignant hypertension and/or a >30% decrease in creatinine clearance.

had rapidly evolving disease (see Table I). Under these circumstances, it would have been expected that cellular and molecular components responsible for the ongoing fibrotic process would be present in the affected tissues. It is possible, however, that by the time dermal fibrosis became clinically apparent, the disease was already well advanced and the very earliest changes may have eluded detection. The third alternative is that, although the TGFβ_1 cDNA employed in these studies would equally detect transcripts for latent or activated TGFβ_1, the absence of hybridization signals in PSS tissues does not exclude the possibility that previously synthesized TGFβ_1 protein either in a latent or active form may be present in PSS tissues. In marked contrast to the negative findings for the expression of TGFβ_1 mRNA in samples from PSS patients, we could readily detect transcripts for this growth factor in affected tissues of patients with DF and GM. These findings suggest the possibility that the pathogenetic mechanisms involved in the fibrotic skin lesions of PSS may be different from those in DF and GM. However, further studies will be necessary to determine precisely the role that TGFβ_1 or other closely related molecules may play in the initiation or progression of the excessive connective tissue accumulation which is the hallmark of these fibrotic diseases.

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